

- 5 *Saccharomyces cerevisiae* yeast strain with functional expression of a Glut transporter

The invention relates to a strain of the yeast *Saccharomyces cerevisiae* which, owing to deletion of the genomic sequences, no longer synthesizes hexose  
10 transporters and, as a consequence, can no longer grow on substrates with hexoses as the only carbon source, and whose ability of growing on a substrate with a hexose as the only carbon source is restored when it expresses a GLUT4 gene.

- 15 Most of the heterotrophic cells transport glucose into the interior of the cell via specific transporter proteins. In the various organisms, different mechanisms which mediate glucose transport have evolved, that is to say proton symport systems, Na<sup>+</sup> glucose cotransporters, binding-protein-dependent systems, phosphotransferase systems and systems for facilitated diffusion. In the case of  
20 eukaryotes, a glucose transporter family, encoded, in mammals, by the *GLUT* genes and in *Saccharomyces cerevisiae* by the *HXT* genes, mediates glucose uptake via facilitated diffusion. These transporters belong to a larger sugar transport superfamily and are characterized by the presence of 12 transmembrane helices and a plurality of conserved amino acid residues and  
25 motifs.

Glucose transport in mammals was the focus of a number of studies since the knowledge of the processes is highly important in diseases associated with deficient glucose homeostasis, such as, for example, diabetes mellitus or Fanconi-Bickel syndrome. Eight glucose transporters (GLUT1 to GLUT5, GLUT8,  
30 GLUT9/SLC2A9, GLUT9 (GenBank accession No. Y17803)) have so far been identified, all of which contribute to the facilitated uptake of glucose. The key

roles of these transporters include the uptake of glucose into a variety of tissues, their storage in the liver, their insulin-dependent uptake into the muscle cells and adipocytes, and glucose measurement by the pancreatic  $\beta$ -cells.

GLUT1 mediates the transport of glucose into the erythrocytes and across the blood-brain barrier, but is also expressed in many other tissues, while GLUT4 is limited to insulin-dependent tissue, mainly to muscle and fatty tissue. In these insulin-dependent tissues, controlling the targeting of GLUT4 transporters into intracellular compartments or plasma membrane compartments constitutes an important mechanism for regulating glucose uptake. In the presence of insulin, intracellular GLUT4 is redistributed to the plasma membrane in order to facilitate the uptake of glucose. GLUT1 is also expressed in these insulin-dependent tissues, and its distribution in the cell is also affected by insulin, but to a lesser extent. In addition, the relative efficacy with which GLUT1 or GLUT4 catalyzes the transport of sugar is not only determined by the extent to which each transporter is targeted to the cell surface, but also by their kinetic properties.

The fact that different glucose transporter isoforms are coexpressed, and that glucose is metabolized rapidly, has made studies into the role and detailed properties of each glucose transporter isoform in these insulin-dependent tissues a complicated task. Heterologous expression systems such as *Xenopus* oocytes, tissue culture cells, insect cells and yeast cells have been used to solve these problems. However, it emerged that these systems presented difficulties: too weak an activity of the heterologously expressed transporters, intrinsic glucose transporters in these systems, the intracellular retention of many of the transporters, or indeed the production of inactive transporters.

No organism is known as yet which, besides a heterologous and functional Glut4 glucose transport protein, expresses no further hexose transport protein, in particular intrinsic hexose transport protein. This leads to a series of disadvantages in the search for compounds which are capable of modifying the transport properties of the Glut4 protein. Such compounds would be of considerable interest as components of pharmaceuticals since it is known that

Glut4 plays an important role in lowering the glucose concentration in the blood together with insulin and other factors. An organism which expresses a functional Glut4 transport protein would allow the search for compounds which directly affect the Glut4 transporter. The side effects of such compounds would be less pronounced since no signal-factor-mediated side effects would occur. Moreover, handling and providing the material would be greatly facilitated if a yeast strain were available. An object of the invention is therefore to provide a yeast strain which expresses a functional Glut4 protein.

- 10 The invention relates to a strain of the yeast *Saccharomyces cerevisiae* which can no longer grow on substrates with hexoses as the only carbon source, and whose ability of growing on a substrate with a hexose as the only carbon source is restored when it expresses a GLUT4 gene. Such a strain can be generated for example by mutating or deleting the relevant genomic sequences. Hexoses are understood as meaning aldoses having 6 carbon atoms, such as glucose, galactose or mannose, and ketoses having 6 carbon atoms, such as fructose or sorbose.

The invention furthermore relates to a strain of the yeast *Saccharomyces cerevisiae* as described above, this strain comprising a GLUT4 gene.

In the yeast *Saccharomyces cerevisiae*, 17 hexose transporters and additionally three maltose transporters are known, which are capable of transporting hexoses into the yeast if they are expressed strongly enough. A strain is known in which all of the transporters which are capable of taking up hexoses have been removed by deletion. This strain now only comprises the two genes MPH2 and MPH3, which are homologous to maltose transport proteins. The two genes MPH2 and MPH3 are repressed when glucose is present in the medium. The generation and characterization of this yeast strain is described in Wieczorke et al., FEBS Lett. 464, 123 – 128 (1999). This strain is not capable of growing on a substrate with glucose as the only carbon source. Mutants can be selected from

this strain which, on the basis of a suitable vector, functionally express Glut1 (strain hxt fgy1-1). If a plasmid vector which carries a GLUT4 gene under the control of a yeast promoter is transformed into the yeast strain hxt fgy1-1, however, only a very small amount of glucose is transported. The functional expression of Glut4 requires further adaptations of this yeast strain to allow a significant glucose transport by means of Glut4. Such yeast strains which take up glucose into the cells by means of a single glucose transporter Glut4 can be isolated on substrates with glucose as the only carbon source. To this end, a yeast strain hxt fgy1-1, which carries a Glut4 gene under the functional control of a yeast promoter, is transformed. These yeast cells which have been transformed in this manner are plated onto a medium which comprises glucose as the only carbon source and are incubated thereon. After a few days incubation at, for example, 30°C, the growth of individual colonies is observed. One of these colonies is isolated. If the yeast plasmid is removed from this colony, no growth takes place on the medium with glucose as the only carbon source. If a yeast vector, which carries a GLUT4 gene under the functional control of a yeast promoter, is retransformed into this strain, which now no longer comprises vector plasmid, then this strain regains the ability of growing on a medium with glucose as the only carbon source. The generation of a *Saccharomyces cerevisiae* strain which makes possible the uptake of glucose by means of a Glut4 transporter is described in detail in the examples. This strain expresses no yeast hexose transporters and is capable of taking up hexoses, in particular glucose, into the cell by means of a gene for a Glut4 transporter, for example a gene which has been transformed into this strain. Yeast strains with this characteristic have been deposited under the number DSM 14035, DSM 14036 or DSM 14037 at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, (DSMZ) in Braunschweig in compliance with the provisions of the Budapest treaty on the international recognition of the deposit of microorganisms for the purposes of patent procedure (Table 1).

In a preferred embodiment of the *Saccharomyces cerevisiae* strain of the present invention, a GLUT4 gene is expressed under the functional control of a yeast expression promoter. The skilled worker is familiar with suitable yeast expression promoters. They are, for example, the SOD1 promoter (superoxide dismutase),

5 ADH promoter (alcohol dehydrogenase), the promoter for the acid phosphatase gene, the HXT2 promoter (glucose transporter 2), the HXT7 promoter (glucose transporter 7), the GAL2 promoter (galactose transporter) and others. For the purpose of expression, the construct, which consists of a yeast expression promoter and a GLUT4 gene, is part of a yeast vector. To carry out the

10 expression, this yeast vector may be present as a self-replicating particle, independently of the yeast genome, or else be stably integrated into the yeast genome. In principle, suitable yeast vectors are all polynucleotide sequences which are capable of multiplication in a yeast. Yeast vectors which can be used in particular are yeast plasmids or yeast artificial chromosomes. Yeast vectors

15 comprise, as a rule, an origin of replication ( $2\mu$ , ars) for starting replication, and a selection marker which usually consists of an auxotrophism marker or an antibiotic resistance gene. Yeast vectors which are known to the skilled worker are, for example, BM272, pCS19, pEMBCYe23, pFL26, pG6, pNN414, pTV3 or others. In principle, the GLUT4 gene of any species can be expressed. A GLUT4

20 gene from humans, mice or rats is preferably expressed. The polynucleotide and amino acid sequences for Glut4 are accessible, for example, via the following Genbank accessions: M20747 (cDNA; human), EMBL:D28561 (cDNA; rat), EMBL:M23382 (cDNA; mouse), Swissprot:P14672 (protein; human), Swissprot:P19357 (protein; rat) and Swissprot:P14142 (protein; mouse). The

25 GLUT4 gene is especially preferably expressed by means of the vector YEp4H7-HsGlut4 (SEQ ID No. 9). The GLUT4 gene of this vector is of human origin. The skilled worker is familiar with the generation of a yeast vector comprising a GLUT4 gene for expression in cells. The generation of such a vector is described in the examples. A yeast vector comprising a gene for expression is transformed

30 into the yeast for the gene to be expressed. Methods which are suitable for this purpose are, for example, electroporation or the incubation of competent cells by

vector DNA. Transformation is a technique with which the skilled worker is familiar and which serves to introduce foreign DNA, in particular plasmids or vectors, to microorganisms such as yeasts or bacteria. Detailed protocols for the transformation of yeasts, yeast vectors, selection of yeast mutants or the

5 expression of proteins in yeasts are found in the manual "Methods in Yeast Genetics, 1997: A Cold Spring Harbor Laboratory Course Manual; Adams Alison (Edt.); Cold Spring Harbor Laboratory; ISBN: 0-87969-508-0", with which the skilled worker is familiar. The evidence that the GLUT4 gene has been

expressed in a yeast according to the invention can be provided in particular by

10 Northern blotting, Western blotting, glucose uptake studies and glucose conversion studies or other methods. Northern blotting involves applying isolated RNA of the organism to be studied to a support such as, for example,

nitrocellulose and fixing it thereon, followed by incubation of this support, which now contains the RNA of the organism, with radiolabeled or fluorescence-labeled

15 DNA of a GLUT4 polynucleotide sequence. The expression of GLUT4-mRNA in a yeast according to the invention is evidenced by the appearance of black bands. In comparison, no black bands are detected with the RNA of a yeast

which is otherwise identical, but has not been transformed with a GLUT4-containing expression vector. Western blotting involves the evidence of the

20 expressed protein after applying a protein extract of the organism to be studied to a membrane support such as nitrocellulose via antibodies. Antibodies for the

Glut4 protein can be obtained, for example, from Alpha Diagnostic International, Inc., 5415 Lost Lane, San Antonio, TX 78238 USA. The assay systems required

for detecting the bound antibody can also be obtained from this supplier. The

25 expressed Glut4 protein is detected in comparison with a yeast strain which is

otherwise identical, but does not comprise Glut4 protein. When carrying out

glucose uptake studies, radiolabeled glucose is supplied as the only carbon

source to the test organism. In contrast with a control strain, which is otherwise

identical but does not comprise Glut4 transporter, the yeast with Glut4 as the

30 only glucose transporter transports this radiolabeled glucose into the interior of

the cell. Glucose conversion can be tested on nutrient media which comprise

glucose as the only carbon source. In contrast to the control, which is otherwise identical but does not comprise Glut4 transporter, the yeast strain with a Glut4 transport protein as the only glucose transporter is capable of growing in the nutrient medium with glucose as the only carbon source. The skilled worker is

5 familiar with these methods which have just been mentioned. Detailed descriptions are found, for example, in "Current Protocols in Molecular Biology; Edited by: F.M. Ausubel, R. Brent, R. E. Kingston, D. M. Moore, J. G. Seidman, J. A. Smith, K. Struhl; published by: John Wiley & Sons; 2000 (currently updated)".

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The invention preferably relates to a strain of the yeast *Saccharomyces cerevisiae* wherein a Glut4 gene from humans, mice or rats is expressed.

The invention especially preferably relates to a strain of the yeast

15 *Saccharomyces cerevisiae* wherein a polynucleotide sequence comprising a coding region of a human Glut4 gene is expressed.

In a further preferred embodiment, the invention relates to one or more strains of the yeast *Saccharomyces cerevisiae* deposited, for example, under the

20 Accession No. DSM 14038, DSM 14039 or DSM 14040 at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig. These strains are listed in table 1. This list contains the information on the yeast strains used, into which the plasmids were transformed, on the plasmids and on the growing conditions for these yeasts.

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The present invention also relates to the generation of a strain of the yeast *Saccharomyces cerevisiae* according to the invention, which is obtainable by the following process steps:

a) Providing a yeast which can no longer grow on substrates with hexoses as the

30 only carbon source;

b) Transformation of the yeast of a) by a plasmid encompassing a GLUT4 gene which is under the functional control of a promoter which can be expressed in yeast;

c) Plating a strain which has been transformed in accordance with b) onto a medium comprising a hexose as the only carbon source;

d) Selection of a strain which has been plated in accordance with c) and which grows on this medium and supports the uptake of hexoses by means of the Glut-4 gene.

The invention also relates to growing such a strain.

To provide a yeast in accordance with the invention, a strain of a yeast *Saccharomyces cerevisiae* which can no longer grow on substrates with hexoses as the only carbon source, but whose ability of growing on a substrate with a hexose as the only carbon source is restored when it expresses a Glut4 gene, is isolated in a first step. This can be effected by mutating or deleting the relevant genomic sequences which encode the hexose transporters. Providing the yeast furthermore requires growing this yeast. Growing takes place by standard methods of microbiology in suitable media. Suitable media for growing a yeast are, for example, complete media, in particular YPD medium (yeast extract/peptone/dextrose medium) or selective media and others. The yeast cells are grown in these media, separated from the medium by centrifugation after growing, and, for the purposes of the method, suspended in an aqueous medium comprising, inter alia, buffer substances, salts or other additives, to give an aqueous suspension. The skilled worker will find information on growing yeasts in "Methods in Yeast Genetics, 1997: A Cold Spring Harbor Laboratory Course Manual; Adams Alison (Edt.); Cold Spring Harbor Laboratory; ISBN: 0-87969-508-0", which has already been mentioned above.

In a preferred embodiment of providing a yeast strain as described above, a GLUT4 gene under the functional control of a promoter which can be expressed in yeast is used for the transformation. To carry out the transformation, reference



may be made to "Methods in Yeast Genetics", which has already been mentioned above.

A GLUT4 gene which is especially preferably used for the transformation for generating such a yeast strain is a GLUT4 gene from humans, mice or rats.

- 5 Moreover, a GLUT4 gene which is especially preferably used for the transformation is one which is present in a polynucleotide sequence as shown in SEQ ID No. 9 or 10. SEQ ID No. 9 discloses the polynucleotide sequence of the yeast vector Yep4H7-HsGLUT4. This vector comprises a polynucleotide sequence under the functional control of the HXT7 promoter, which
- 10 polynucleotide sequence encodes the amino acid sequence of the human GLUT4 gene. SEQ ID No. 10 comprises the polynucleotide sequence of vector H2rg4g2. The yeast plasmid H2rg4g2 carries a GLUT4 gene of rats under the functional control of an HXT2 promoter. Functional control of the GLUT4 gene by the promoter means that, by means of the promoter, an mRNA is transcribed
- 15 which can be translated into a Glut4 protein. As regards the disclosures of the GLUT4 sequences and the methods used, reference may be made to what has already been said above.

- The invention furthermore relates to a method of identifying a compound which
- 20 increases or reduces the amount of a hexose transported by means of a Glut4 protein, with the following process steps:

- a) Providing a strain of the yeast *Saccharomyces cerevisiae* according to the present invention which expresses a GLUT4 gene;
- b) Determining the amount of a hexose taken up by it;
- 25 c) Providing a compound;
- d) Contacting a strain of the yeast provided in accordance with a) with a compound provided in accordance with c);
- e) Determining the amount of a hexose which is taken up into the yeast strain after contacting in accordance with d);
- 30 f) Identifying a compound which increases or reduces the amount of a hexose transported by means of a Glut4 protein by comparing the amount of the hexose

taken up into the strain before and after contacting in accordance with d), which is determined in accordance with b) and e).

To provide a yeast according to the invention, a strain of a yeast *Saccharomyces cerevisiae*, which can no longer grow on substrates with hexoses as the only carbon source, but whose ability of growing on a substrate with a hexose as the only carbon source is restored when it expresses a GLUT4 gene, is isolated in a first step. This yeast strain is transformed by means of a yeast vector comprising a GLUT4 gene under the functional control of a yeast promoter. The generation of such a yeast strain is described in the examples. Providing the yeast furthermore requires growing this yeast. Growing takes place by standard methods of microbiology in suitable media. Suitable media for growing a yeast are, for example, complete media, in particular YPD medium (yeast extract/peptone/dextrose medium) or selective media and others. The yeast cells are grown in these media, separated from the medium by centrifugation after growing, and, for the purposes of the method, suspended in an aqueous medium comprising, inter alia, buffer substances, salts or other additives, to give an aqueous suspension. The skilled worker will find information on growing yeasts in "Methods in Yeast Genetics, 1997: A Cold Spring Harbor Laboratory Course Manual; Adams Alison (Edt.); Cold Spring Harbor Laboratory; ISBN: 0-87969-508-0", which has already been mentioned above.

The amount of a hexose which is taken up by a yeast strain provided as has just been described above can be determined by uptake studies with radiolabeled glucose. To this end, a specific amount of yeast cells, for example an amount with a wet weight of 60 mg per ml, is suspended in, for example, 100 µl of a buffer and treated with a defined amount of  $^{14}\text{C}$ - or  $^3\text{H}$ -labeled glucose as the only carbon source. The cells are incubated, and defined amounts of the cells are sampled at specific times. The amount of glucose taken up is determined with the aid of LSC (liquid scintillation counting). However, the amount of a hexose which is taken up by a yeast strain provided as has just been described

above can also be determined by growth tests on media with glucose as the only carbon source. To this end, the growth rate of the strain is determined after addition of the compound, for example by regularly measuring the optical density of the culture at 600 nm, and this value is compared with the growth rate of a control strain (for example yeast wild-type strain).

Providing a compound is done in particular by chemical synthesis, or by isolating chemicals from biological organisms. Chemical synthesis may also be automated. The compounds obtained by synthesis or isolation can be dissolved in a suitable solvent. Suitable solvents are, in particular, aqueous solutions comprising a particular amount of an organic solvent such as, for example, DMSO (dimethyl sulfoxide).

Contacting a strain of the yeast with a compound for identifying a compound which increases or reduces the amount of a hexose transported by means of a Glut4 protein is done in particular in automated laboratory systems provided for this purpose. Such systems can be composed of specifically prepared chambers with recesses, of microtiter plates, Eppendorf tubes or laboratory glassware. Automated laboratory systems are generally designed for high throughput rates. A process like what has just been described which is carried out with the aid of an automated laboratory system is therefore also referred to as HTS (high throughput screening).

After contacting the yeast with the compound, the amount of a hexose, in particular glucose, which is transported by the yeast cell into the interior of the cell under these conditions is determined. To this end, the same procedure may be used which has already been described for determining the glucose uptake for a strain which has not been contacted with a compound.

The identification of a compound which increases or reduces the amount of a hexose transported by means of a Glut4 protein is carried out by comparing the

amount of the hexose taken up into the strain before and after contacting it with the compound.

The invention furthermore relates to a pharmaceutical comprising a compound which has been identified and, if appropriate, further developed by the method which has just been described using the Glut4 gene, and to adjuvants for formulating the pharmaceutical for the treatment of diabetes or adiposity. The further development of a compound which has been identified means that, firstly, the specificity with regard to the target protein, in this case Glut4, is improved, secondly that the availability in the animal or human organism is increased and thirdly that any existing undesired side effects are reduced. To this end, a series of methods is available to the skilled worker, including, for example but not by way of limitation, the use of pharmacological animal models such as diabetic rats or ob/ob mice, the use of biochemical in-vitro measurements, the use of virtual structure models of compounds and of the Glut4 protein. Adjuvants for the formulation of a pharmaceutical make possible the conditioning of the active substance with the purpose of tailoring the application, distribution and development of action of the active ingredient to the application in question. Examples of such adjuvants are fillers, binders, disintegrants or glidants such as lactose, sucrose, mannitol, sorbitol, cellulose, starch, dicalcium phosphate, polyglycols, alginates, polyvinylpyrrolidone, carboxymethylcellulose, talc or silicon dioxide.

Diabetes is evidenced by the excretion of glucose together with the urine combined with an abnormal increase in the blood glucose level (hyperglycemia) owing to a chronic metabolic condition due to lack of insulin or a reduced insulin effect. The lack of, or reduced, insulin effect leads to incomplete absorption and conversion of the glucose taken up into the blood by the body cells. In fatty tissue, insulin-antagonistic hormones have the effect of increasing lipolysis, which entails an increase in the free fatty acid levels in the blood.

Adiposity (obesity) is the abnormal weight gain owing to an energy imbalance due to excessive intake of calories, which constitutes a health hazard.

The invention furthermore relates to the use of a compound which has been identified, and, if appropriate, further developed by a method using the Glut4 protein for the preparation of a pharmaceutical for the treatment of diabetes or adiposity. Pharmaceuticals are dosage forms of pharmacologically active substances for the therapy of diseases or bodily malfunctions in humans and animals. For example, powders, granules, tablets, pills, lozenges, sugar-coated tablets, capsules, liquid extracts, tinctures and syrups are known for oral therapy. Examples which are used for external administration are aerosols, sprays, gels, ointments or powders. Injectable or infusible solutions allow parenteral administration, with vials, bottles or prefilled syringes being used. These and other pharmaceuticals are known to the skilled worker in the field of pharmaceutical technology.

The invention furthermore relates to a method of identifying a compound which increases or reduces the amount of a hexose transported by means of a Glut1 protein, with the following process steps:

- a) Providing a strain of the yeast *Saccharomyces cerevisiae* which can no longer grow on substrates with hexoses as the only carbon source and whose ability of growing on a substrate with a hexose as the only carbon source is restored when it expresses a GLUT1 gene, this strain comprising a GLUT1 gene under the functional control of a promoter which can be expressed in yeast;
- b) Determining the amount of a hexose which is taken up into this strain provided in accordance with a);
- c) Providing a compound;
- d) Contacting a strain of the yeast provided in accordance with a) with a compound provided in accordance with c);
- e) Determining the amount of a hexose which is taken up into the yeast strain after contacting in accordance with d);

f) Identifying a compound which increases or reduces the amount of a hexose transported by means of a Glut1 protein by comparing the amount of the hexose taken up into the strain before and after contacting in accordance with d), which is determined in accordance with b) and e).

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To provide a yeast according to the invention, a strain of a yeast *Saccharomyces cerevisiae* which no longer forms hexose transporters owing to deletion of the genomic sequences and, as a consequence, is no longer capable of growing on substrates with hexoses as the only carbon source, but whose ability of growing on a substrate with a hexose as the only carbon source is restored when it expresses a GLUT1 gene, is isolated in a first step. Such strains are deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH under the numbers DSM 14031, DSM 14032 or DSM 14034.

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In the yeast *Saccharomyces cerevisiae*, 17 hexose transporters and additionally three maltose transporters are known, which are capable of transporting hexoses into the yeast. A strain is known in which all of the transporters which are capable of taking up hexoses have been removed by deletion. The generation and characterization of this yeast strain is described in Wieczorke et al., FEBS Lett. 464, 123 – 128 (1999). This strain is not capable of growing on a substrate with a hexose as the only carbon source. If a plasmid vector which carries a Glut1 gene under the control of a yeast promoter is transformed into such a yeast strain, no glucose transport takes place nevertheless. The functional expression of Glut1 requires further adaptations of this yeast strain in order to make possible the transport of glucose by means of Glut1. Such yeast strains which take up glucose into the cells by means of a single glucose transporter Glut1 can be isolated on substrates with glucose as the only carbon source. To this end, a yeast strain which no longer expresses intact hexose-transporting proteins is transformed with a yeast vector which carries a GLUT1 gene under the functional control of a yeast promoter. These yeast cells which have been transformed in this manner are plated onto a medium comprising glucose as the only carbon

source and are incubated thereon. After a few days incubation at, for example, 30°C, the growth of individual colonies is observed. One of these colonies is isolated. If the yeast plasmid is removed from this colony, no growth takes place on the medium with glucose as the only carbon source. If a yeast vector carrying

5 a GLUT1 gene under the functional control of a yeast promoter is now transformed into this strain, which no longer comprises vector plasmid, then this strain regains the ability of growing on a medium with glucose as the only carbon source.

- 10 To transform a yeast strain, a GLUT1 gene from humans, mice or rats is used in particular. Polynucleotide sequences and amino acid sequences for Glut1 are disclosed under the following code numbers of the databases indicated:  
 EMBL:M20653 (cDNA; human), EMBL:M13979 (cDNA; rat), EMBL:M23384 (cDNA; mouse), Swissprot:P11166 (protein; human), Swissprot:P11167 (protein; rat), Swissprot:P17809 (protein; mouse).
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- The generation of such a yeast strain is described in the examples. Providing the yeast furthermore requires growing this yeast. Growing takes place by standard methods of microbiology in suitable media. Suitable media for growing a yeast
- 20 are, for example, complete media, in particular YPD medium (yeast extract/peptone/dextrose medium) or selective media. The yeast cells are grown in these media, separated from the medium by centrifugation after growing, and, for the purposes of the method, suspended in an aqueous medium comprising, inter alia, buffer substances, salts or other additives, to give an aqueous
- 25 suspension. The skilled worker will find information on growing yeasts in "Methods in Yeast Genetics, 1997: A Cold Spring Harbor Laboratory Course Manual; Adams Alison (Edt.); Cold Spring Harbor Laboratory; ISBN: 0-87969-508-0", which has already been mentioned above.

- 30 In a preferred embodiment of this method, a strain of the yeast *Saccharomyces cerevisiae* is provided which comprises a GLUT1 gene under the functional

control of a promoter which can be expressed in yeast. Such strains which are suitable for this method were deposited at the Sammlung für Mikroorganismen und Zellkulturen GmbH under the number DSM 14033, DSM 14026 or DSM 14033.

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A GLUT1 gene as constituent of a plasmid is disclosed in SEQ ID No. 11 or SEQ ID No. 12. SEQ ID No. 11 comprises the sequence of the yeast vector Yep4H7-HsGlut1. This plasmid comprises the polynucleotide sequence of a human GLUT1 gene under the functional control of an HXT7 promoter. SEQ ID No. 12  
10 comprises the polynucleotide sequence of the yeast vector H2rg1g2. This plasmid comprises the polynucleotide sequence of a GLUT1 gene from rats under the functional control of the HXT2 promoter.

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The amount of a hexose which is taken up by a yeast strain provided as has just been described above can be determined by uptake studies with radiolabeled glucose. To this end, a specific amount of yeast cells, for example an amount with a wet weight of 60 mg, is suspended in, for example, 100 µl of a buffer and treated with a defined amount of  $^{14}\text{C}$ - or  $^3\text{H}$ -labeled glucose as the only carbon source. The cells are incubated, and defined amounts of the cells are sampled at  
20 specific times. The amount of glucose taken up is determined with the aid of LSC (liquid scintillation counting).

Providing a compound is done in particular by chemical synthesis, or by isolating chemicals from biological organisms. Chemical synthesis may also be  
25 automated. The compounds obtained by synthesis or isolation can be dissolved in a suitable solvent. Suitable solvents are, in particular, aqueous solutions comprising a particular amount of an organic solvent such as, for example, DMSO (dimethyl sulfoxide).

30 Contacting a strain of the yeast with a compound for identifying a compound which increases or reduces the amount of a hexose transported by means of a



Glut1 protein is done in particular in automated laboratory systems provided for this purpose. Such systems can be composed of specifically prepared chambers with recesses, of microtiter plates, of Eppendorf tubes or of laboratory glassware. Automated laboratory systems are generally designed for high throughput rates.

- 5 A process like what has just been described which is carried out with the aid of an automated laboratory system is therefore also referred to as HTS (high throughput screening).

- 10 After contacting the yeast with the compound, the amount of a hexose, in particular glucose, which is transported by the yeast cell into the interior of the cell under these conditions is determined. To this end, the same procedure may be used which has already been described for determining the glucose uptake for a strain which has not been contacted with a compound.
- 15 The identification of a compound which increases or reduces the amount of a hexose transported by means of a Glut1 protein is carried out by comparing the amount of the hexose taken up into the strain before and after contacting it with the compound.
- 20 The invention furthermore relates to a pharmaceutical comprising a compound which has been identified and, if appropriate, further developed by the process which has just been described using the Glut1 gene, and to adjuvants for formulating the pharmaceutical for the treatment of diabetes or adiposity. The further development of a compound which has been identified means that, firstly,- 25 the specificity with regard to the target protein, in this case Glut4, is improved, secondly that the availability in the animal or human organism is increased and thirdly that any existing undesired side effects are reduced. To this end, a series of methods is available to the skilled worker, including, for example but not by way of limitation, the use of pharmacological animal models such as diabetic rats
- 30 or ob/ob mice, the use of biochemical in-vitro measurements, the use of virtual structure models of compounds and of the Glut1 protein. Adjuvants for the

formulation of a pharmaceutical make possible the conditioning of the active substance with the purpose of tailoring the application, distribution and development of action of the active ingredient to the application in question. Examples of such adjuvants are fillers, binders, disintegrants or glidants such as

5 lactose, sucrose, mannitol, sorbitol, cellulose, starch, dicalcium phosphate, polyglycols, alginates, polyvinylpyrrolidone, carboxymethylcellulose, talc or silicon dioxide.

Diabetes is evidenced by the excretion of glucose together with the urine

10 combined with an abnormal increase in the blood glucose level (hyperglycemia) owing to a chronic metabolic condition due to lack of insulin or a reduced insulin effect. The lack of, or reduced, insulin effect leads to incomplete absorption and conversion of the glucose taken up into the blood by the body cells. In fatty tissue, insulin-antagonistic hormones have the effect of increasing lipolysis,

15 which entails an increase in the free fatty acid levels in the blood. Adiposity (obesity) is the abnormal weight gain owing to an energy imbalance due to excessive intake of calories, which constitutes a health hazard.

The invention furthermore relates to the use of a compound which has been identified and, if appropriate, further developed by a method using the Glut1

20 protein for the preparation of a pharmaceutical for the treatment of diabetes or adiposity. Pharmaceuticals are dosage forms of pharmacologically active substances for the therapy of diseases or bodily malfunctions in humans and animals. For example, powders, granules, tablets, pills, lozenges, sugar-coated

25 tablets, capsules, liquid extracts, tinctures and syrups are known for oral therapy. Examples which are used for external administration are aerosols, sprays, gels, ointments or powders. Injectable or infusible solutions allow parenteral administration, with vials, bottles or prefilled syringes being used. These and other pharmaceuticals are known to the skilled worker in the field of

30 pharmaceutical technology.

The invention furthermore relates to the polynucleotide sequence of SEQ ID No. 13 and to the polynucleotide sequence of SEQ ID No. 14. The polynucleotide sequences of SEQ ID No. 13 and 14 encode mutations of the rat Glut1 gene which lead to the substitution of individual amino acids in the protein in question.

- 5 The polynucleotide sequence of SEQ ID No. 13 encodes a Glut1 protein with a substitution of valine with methionine in position 69 of the amino acid chain. The polynucleotide sequence of SEQ ID No. 14 encodes a Glut1 protein where an alanine is substituted with methionine in position 70 of the amino acid chain. Both protein mutants support the uptake of glucose even in a strain whose hexose  
10 transporters have been switched off by deletion, but which does not yet support the uptake of glucose by the wild-type Glut1 protein. Such mutants can be obtained for example via selection for suppressor mutations or via in-vitro mutagenesis.

- The invention furthermore relates to a Glut1 protein which is encoded by the  
15 polynucleotide sequence of SEQ ID No. 13 or 14.

- The invention also relates to yeast strains comprising a polynucleotide sequence of SEQ ID No. 13 or a polynucleotide sequence of SEQ ID No. 14. Such yeast strains are deposited at the Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH as DSM 14026 and DSM 14027. To generate these strains,  
20 yeast vectors corresponding to SEQ ID No. 13 or 14 are transformed into a yeast strain which is no longer capable of growth on substrates with hexoses as the only carbon source and whose ability of growing on a substrate with hexose as the only carbon source is eventually restored when a Glut1 gene is expressed in this strain. Then, after transformation, the cells are plated onto a medium with  
25 glucose as the only carbon source. The colonies growing on this medium are isolated. The yeast strain which has been transformed in this manner is suitable for example for carrying out a method of identifying a compound which increases or reduces the amount of a hexose transported by means of a Glut1 protein.

- 30 Abbreviations

HXT hexose transporter  
 ORF open reading frame  
 PCR polymerase chain reaction

5

## Examples

### Growing the yeast strains

- 10 All of the yeast strains described herein were derived from strain CEN.PK2-1C (*MATa leu2-3, 112 ura3-52 trp1-289 his3-Δ1 MAL2-8<sup>c</sup> SUC2*). The generation of a yeast strain with deletions in the hexose transporter genes (*HXT*) was described by Wieczorke et al., FEBS Lett. 464, 123 - 128 (1999): EBY.18ga (*MATa Δhxt1-17 Δgal2 Δagt1 Δstl1 leu2-3, 112 ura3-52 trp1-289 his3-Δ1 MAL2-8<sup>c</sup> SUC2*), EBY.VW4000 (*MATa Δhxt1-17 Δgal2 Δagt1 Δmph2 Δmph3 Δstl1 leu2-3, 112 ura3-52 trp1-289 his3-Δ1 MAL2-8<sup>c</sup> SUC2*). The media were based on 1% yeast extract and 2% peptone (YP), while the minimal media were composed of 0.67% Difco yeast nitrogen base without amino acids (YNB) and comprised additives for auxotrophism requirements and different carbon sources.
- 20 The yeast cells were grown aerobically at 30°C on an orbital shaker or on Agar plates. Cell growth was monitored by measuring the optical density at 600 nm (OD<sub>600</sub>).

### 25 Glucose uptake determination

- The glucose transport was measured as the uptake of D-[U-<sup>14</sup>C]-glucose (Amersham), and the kinetics parameters were determined from Eadie-Hofstee graphs. The cells were spun, washed with phosphate buffer and resuspended in phosphate buffer at a concentration of 60 mg (fresh weight) per ml. Glucose uptake was determined at glucose concentrations of between 0.2 and 100 mM,
- 30

and the specific activity of the substrate ranged between 0.1 and 55.5 kBq  $\mu\text{mol}^{-1}$ .

1. Cells and glucose solutions were preincubated for 5 minutes at 30°C. Glucose uptake was started by treating the cells with radioactive glucose. After incubation for 5 seconds, 10 ml of ice-cold stop buffer (0.1 M  $\text{K}_2\text{PO}_4$ , pH 6.5, 500 mM glucose) were added, and the cells were filtered rapidly on glass fiber filters ( $\varnothing=24$  mm, Whatman). The filters were washed rapidly three times with ice-cold buffer, and the radioactivity incorporated was measured in a liquid scintillation counter. Inhibition by cytochalasin B (final concentration 20  $\mu\text{M}$ , dissolved in ethanol) was measured in a 15-second-uptake test with 50 mM or 100 mM radioactive glucose after incubating the cells for 15 minutes in the presence of the inhibitor or only of the solvent.

Construction of H2rg4g2 (SEQ ID No. 10) and H2rg1g2 (SEQ ID No. 12)

- H2rg4g2 and H2rg1g2 are DNA constructs comprising an HXT2 promoter (promoter of the yeast glucose transport protein 2) linked functionally to a GLUT4 gene (in SEQ ID No. 10) or GLUT1 gene (in SEQ ID No. 12) from rats. An 0.5 kb *SalI/EcoRI*-GAL2 promoter fragment of plasmids GLUT1-pTV3 and GLUT4-pTV3 (Kasahara and Kasahara, *Biochem J.* 315, 177 – 182 (1996); Kasahara and Kasahara, *Biochim. Biophys. Acta* 857, 146 – 154 (1997)) was substituted in each case for an 0.5 kb DNA fragment comprising the yeast *HXT2* promoter from –452 bp through +9 bp (Genbank: P23585).

- Construction of YEp4H7-HsGLUT1 (SEQ ID No. 11) and YEp4H7-HsGLUT4 (SEQ ID No. 9)

- YEp4H7-HsGLUT1 and YEp4H7-HsGLUT4 are plasmids in which a promoter fragment of positions –392 through –1 of the HXT7 promoter (promoter of the HXT7 gene) is linked functionally to a human GLUT1 gene (in SEQ ID No. 11) or GLUT4 gene (in SEQ ID No. 9). The fragment of the promoter was used since the complete HXT7 promoter is repressed by glucose.

A 0.4 kb *SacI/Spel*-*MET25* promoter fragment from p426*MET25* (Mumberg et al., Nucleic Acids Res. 22, 5767 – 5768 (1994)) was substituted with an 0.4 kb DNA fragment comprising an *HXT7* promoter fragment from positions -392 through – 1, which had been amplified by means of PCR from an *HXT7* gene

(Genbank:P39004) as template, using the primers P426H7-1 (SEQ ID No. 1) and P426H7-2 (SEQ ID No. 2), giving rise to plasmid YEp4H7 (SEQ ID No. 15). The human GLUT1 and GLUT4 ORFs (open reading frames) were amplified over 10 cycles by means of PCR with the primer pairs HSG1-F7/T2-HSG1 (SEQ ID No. 3.4) for Glut1 and HSG4-F7/T2-HSG4 (SEQ ID No. 5.6) for Glut4 and a human GLUT1 (EMBL:M20653) and human GLUT4 cDNA (Genbank:M20747) as templates. The PCR products were reamplified over 10 cycles using the primers T71-ORF (SEQ ID No. 7) and T2-HSG1 (SEQ ID No. 4) or T2-HSG4 (SEQ ID No. 6). Upstream and downstream of the GLUT ORF sequences, the PCR end products contain sequences which are homologous to the *HXT7* promoter region or the *CYC1* termination region (iso-cytochrome c1) of plasmid YEp4H7. They were transformed into the yeast strain EBY.F4-1 together with the *EcoRI*-linearized YEp4H7, whereupon, following homologous recombination in yeast, the transformation products were selected for uracil prototrophism in a 2% strength maltose medium.

Expression of GLUT1 and GLUT4 from rats in a hexose-transport-deficient yeast strain

The yeast multicopy expression plasmids GLUT1-pTV3e and GLUT4-pTV3e carry the glucose transporter genes GLUT1 and GLUT4 from rats under the control of the galactose-inducible and glucose-repressible yeast *GAL2* promoter. In the two constructs, the *GAL2* promoter was replaced by the glucose-inducible yeast *HXT2* promoter. These vectors were transformed into the yeast strain EBY.18ga ( $\Delta hxt$ ), which is not capable of taking up any hexoses whatsoever and can therefore not grow on media with glucose or other hexoses as the only carbon source. The cells were plated onto a tryptophan-free synthetic medium

with maltose as carbon source. The transformants were plated onto the same basal medium without maltose, but with different glucose concentrations (5 mM, 10 mM, 50 mM, 100 mM), using the replica-plating method. The transformants did not grow on the different glucose media, not even when incubated for up to

5 one week at 30°C. This proves that the Glut1 and Glut4 glucose transporters do not support glucose uptake in a normal *S. cerevisiae* strain.

#### Glucose uptake via the Glut1 transporter in yeast cells

10 It emerged that, following prolonged incubation of Glut1 transformants of strain EBY.18ga on a glucose medium, colonies (termed suppressor mutants or suppressor colonies hereinbelow) grew, and these were obviously capable of taking up, and converting, glucose. The GLUT1 and GLUT4 transformants were therefore plated onto agar plates with a YNB medium with 10 mM of glucose as

15 the only carbon source. Following irradiation with UV light at a sublethal dose, the cells were incubated for 7-14 days at 30°C. While no suppressor colonies were observed in the case of the GLUT4 transformants, several suppressor colonies which were capable of growing on glucose grew on the agar plates with the GLUT1 transformants. Several of the GLUT1 suppressor mutants were

20 grown over 15 generations in nonselective YP maltose medium. All cells which had lost their plasmids were no longer capable of growing on media with glucose as the carbon source. It was thus possible to demonstrate that growth on glucose as the only carbon source was GLUT1-dependent. After the retransformation of the original wild-type H2rg1g2 plasmid into these cells, one of several yeast

25 strains regains the ability of growing on glucose. This confirms that this strain comprises, in its genome, a mutation which eliminates the inhibitory action on functional GLUT1 expression. The mutated allele was termed *fgy1-1* (which stands for "functional expression of Glut1 in yeast"), and the strain was termed EBY.S7.

H2rg1g2 plasmids were isolated from other suppressor mutants, amplified in *E. coli* and transformed back into the original glucose-transport-deficient yeast strain EBY.18ga ( $\Delta hxt$ ). Several of these plasmids allowed growth on a synthetic medium with 10 mM glucose as the only carbon source. Accordingly, these

5 GLUT1 sequences comprised mutations which converted the corresponding GLUT1 protein in the yeast into a functional glucose transporter. For example, such mutants comprised a substitution of valine with methionine at the position of amino acid 69 (SEQ ID No.13) or a substitution of alanine with methionine at the position of amino acid 70 (SEQ ID No. 14). The mutant of SEQ ID No. 13 was  
10 found during screening of the mutants as described above. The mutant of SEQ ID No. 14 was obtained by in-vitro mutagenesis as shown hereinbelow. The principle of the in-vitro mutagenesis method applied is described by Boles and Miosga (1995) (Boles and Miosga, Curr Genet. 28, 197 – 198 (1995)). In a first PCR reaction, plasmid YEph2-rGLUT1 (20 ng) as DNA template was employed together with the primers seqhxt2 (SEQ ID No. 16) and glutmet2 (SEQ ID No. 17) (in each case 100 pmol) (PCR conditions: 95°C 45 sec, 50°C 30 sec, 72°C 2 min, 25 cycles, taq polymerase). The primer glutmet2 contains a base sequence which is modified over the normal GLUT1 gene and which leads to a substitution of alanine with methionine at the position of amino acid 70 of GLUT1 from rats.  
15 The resulting PCR fragment was purified by agarose gel electrophoresis followed by gel extraction. In a second PCR reaction, the purified PCR fragment (20 ng) was used together with plasmid GLUT1-pTV3 (Kasahara and Kasahara, Biochem J. 315, 177 – 182 (1996)) as DNA template (50 ng) and together with the primers seqhxt2 and seq2gal2 (SEQ ID No. 18) (in each case 100 pmol)  
20 (PCR conditions: 95°C 45 sec, 54°C 30 sec, 72°C 2 min, 20 cycles, taq polymerase). Since the primer seqhxt2 only binds to the fragment of the first PCR reaction, only those DNA sequences were amplified in this second PCR reaction which lead to a substitution of alanine with methionine at the position of amino acid 70. The resulting PCR fragment with the mutated GLUT1 gene was purified  
25 by means of agarose gel electrophoresis followed by gel extraction, and exchanged for the wild-type GLUT1 gene in plasmid YEph2-rGLUT1. This  
30



plasmid (SEQ ID No. 14) was transformed into the glucose-transport-deficient yeast strain EBY.18ga ( $\Delta hxt$ ) and allowed growth on a synthetic medium with glucose as the only carbon source.

- 5 Strains of the yeast *Saccharomyces cerevisiae*, which take up glucose via the Glut4 transporter

Strain EBY.S7 ( $\Delta hxt fgy1-1$ ) apparently comprises a genome mutation, viz. *fgy1-1*, which allows Glut1 to become functional in yeast and to support the  
 10 uptake of glucose across the plasma membrane into the cells.

Following transformation of strain EBY.S7 ( $\Delta hxt fgy1-1$ ) with H2rg4g2, suppressor colonies were isolated which were capable of growth on media with glucose as the only carbon source.

15 Nine of these GLUT4 suppressor mutants were grown for over 15 generations in nonselective YP maltose medium. All cells which had lost their plasmids were likewise no longer capable of growing on 10 mM glucose media, which confirms that the earlier growth was GLUT4-dependent. The H2rg4g2 plasmids were  
 20 reisolated from the nine suppressor strains, amplified in *E. coli* and transformed back into the original glucose-transport-deficient yeast strain EBY.S7. None of the plasmids allowed growth on a synthetic medium with 10 mM glucose as the only carbon source. This demonstrated that they comprised no "activated" mutant forms of GLUT4. Following retransformation of the original wild-type H2rg4g2  
 25 plasmid into the nine, now plasmid-free, suppressor strains, all of these strains regained the ability of growing on glucose, as opposed to transformants which comprised a control vector without Glut4 transport protein gene. The corresponding mutations of this strain were termed *fgy4-X* ( $x = 1 - 9$ ). The mutated alleles *fgy4-X* caused functional GLUT4 expression of a GLUT4 gene  
 30 expressed in these strains. The object of the invention was herewith achieved.

An overview of the yeast strains according to the invention which have been used is found in the table.

- The table gives an overview of the yeast strains used in the present invention
- 5 including the genotype, the growth conditions required for growing them, and the respective deposit number at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

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List of strains

No.	Strain	Genotype	Phenotype	Plasmid	DSM-No.
1	EBY.18ga	<i>MATa Δhxt1-17Δgal2 Δagt1 Δstl1 leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8<sup>c</sup> SUC2</i>	Grows with 1% maltose as C-source; auxotrophic for glucose, leucine, tryptophan, histidine and uracil	-----	DSM 14031
2	EBY.18ga	<i>MATa Δhxt1-17Δgal2 Δagt1 Δstl1 leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8<sup>c</sup> SUC2</i>	Grows with 0.2% glucose or 1% maltose as C source; auxotrophic for leucine, histidine and uracil	YEpH2-rGLUT1V69M (selection marker TRP1)	DSM 14026
3	EBY.18ga	<i>MATa Δhxt1-17Δgal2 Δagt1 Δstl1 leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8<sup>c</sup> SUC2</i>	Grows with 0.2% glucose or 1% maltose as C source; auxotrophic for leucine, histidine and uracil	YEpH2-rGLUT1A70M (selection marker TRP1)	DSM 14027
4	EBY.S7	<i>MATa Δhxt1-17 Δgal2 Δagt1 Δstl1 fgy1-1 leu2-3,112 ura3-52 trp1-28,9 his3-Δ1 MAL2-8<sup>c</sup> SUC2</i>	Grows with 1% maltose as C-source; auxotrophic for glucose, leucine, tryptophan, histidine and uracil	-----	DSM 14032
5	EBY.S7	<i>MATa Δhxt1-17 Δgal2 Δagt1 Δstl1 fgy1-1 leu2-3,112 ura3-52 trp1-28,9 his3-Δ1 MAL2-8<sup>c</sup> SUC2</i>	Grows with 0.2% glucose or 1% maltose as C source; auxotrophic for leucine, tryptophan and histidine	YEp4H7-HsGLUT1 (selection marker URA3)	DSM 14033
6	EBY.VW4000	<i>MATa Δhxt1-17 Δgal2 Δagt1 Δstl1 Δmph2 Δmph3 leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8<sup>c</sup> SUC2</i>	Grows with 1% maltose as C-source; auxotrophic for glucose, leucine, tryptophan, histidine and uracil	-----	DSM 14034
7	EBY.f4-1	<i>MATa Δhxt1-17 Δgal2 Δagt1 Δstl1 fgy4-1 fgy4-1 leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8<sup>c</sup> SUC2</i>	Grows with 1% maltose as C-source; auxotrophic for glucose, leucine, tryptophan, histidine and uracil	-----	DSM 14035
8	EBY.f4-4	<i>MATa Δhxt1-17 Δgal2 Δagt1 Δstl1 fgy4-4 fgy4-1 leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8<sup>c</sup> SUC2</i>	Grows with 1% maltose as C-source; auxotrophic for glucose, leucine, tryptophan, histidine and uracil	-----	DSM 14036
9	EBY.f4-7	<i>MATa Δhxt1-17 Δgal2 Δagt1 Δstl1 fgy4-7 fgy4-1 leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8<sup>c</sup> SUC2</i>	Grows with 1% maltose as C-source; auxotrophic for glucose, leucine, tryptophan, histidine and uracil	-----	DSM 14037
10	EBY.f4-1	<i>MATa Δhxt1-17 Δgal2 Δagt1 Δstl1 fgy4-1 fgy4-1 leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8<sup>c</sup> SUC2</i>	Grows with 0.2% glucose or 1% maltose as C source; auxotrophic for leucine, tryptophan and histidine	YEp4H7-HsGLUT4 (selection marker URA3)	DSM 14038
11	EBY.f4-4	<i>MATa Δhxt1-17 Δgal2 Δagt1 Δstl1 fgy4-4 fgy4-1 leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8<sup>c</sup> SUC2</i>	Grows with 0.2% glucose or 1% maltose as C source; auxotrophic for leucine, tryptophan and histidine	YEp4H7-HsGLUT4 (selection marker URA3)	DSM 14039

Table

12	EBY-f4-7	<i>MATa Δhxt1-17 Δgal2 Δagl1 Δstl1 fgy4-7 fgy4-1 leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8<sup>c</sup> SUC2</i>	Grows with 0.2% glucose or 1% maltose as C source; auxotrophic for leucine, tryptophan and histidine	YE <sub>p</sub> 4H7-HISGLUT <sub>4</sub> (selection marker URA3)	DSM 14040
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Basal medium: 0.67% yeast nitrogen base without amino acids (Difco); pH 6.2. Supplementation of the auxotrophisms: leucine (0.44 mM), tryptophan (0.19 mM), histidine (0.25 mM), uracil (0.44 mM). Maltose can be employed at a concentration of between 1 and 2%; glucose between 0.2 and 2% (better growth at lower concentrations).

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